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Neutral Proteinase Activity in Skeletal Muscle from Thermally Injured Rats[1,2]

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Male Sprague–Dawley rats that received 60% total body surface, full-thickness, scald burns on the dorsum and abdomen were used in this study. Neutral proteinase and Ca^{2+}-activated neutral proteinase activities were measured in gastrocnemius and soleus muscles at 3 and 21 days after the thermal injury. Neutral proteinase activity decreased significantly in the soleus (50%) and gastrocnemius (46%) muscles on the third postburn day. Ca^{2+}-activated neutral proteinase was unchanged at this time. Neutral proteinase and Ca^{2+}-activated neutral proteinase activities were unaltered at 21 days postinjury. These results may reflect a protein-sparing effect on the third postburn day which could be an early intracellular change prior to an increase in selected enzyme proteins during the hypermetabolic phase after thermal injury.

INTRODUCTION

Subcellular muscle proteins are known to turn over at different rates [20, 21], but the role of proteinases in skeletal muscle protein degradation is unclear. The best characterized proteinases are the cathepsins, which are lysosomal proteinases that are active over acidic pH ranges [1, 11]. However, these proteinases are not highly substrate specific and are not generally active within a neutral pH range [5, 19]. These considerations cast doubt on the role of cathepsins in protein degradation *in vivo*. Several investigators have identified a proteinase active at alkaline pH in rat muscle [9, 18]. It has been postulated that this proteinase is of mast cell origin and is essentially the same enzyme identified as mast cell alkaline protease [17]. It is doubtful that this proteinase is involved in intracellular muscle protein degradation because of its extracellular location and pH optimum. Neutral proteinases active at near neutral pH values have been identified in skeletal muscle [2, 4, 10, 15]. These proteinases are extralysosomal, and their proteolytic activity is low in comparison to acid and alkaline proteinases [5]. Within this category of proteinases is the calcium-activated neutral proteinase. This proteinase requires calcium ions for activity and degrades the Z disk of myofibrils specifically [2, 4]. Neutral proteinases and calcium-activated neutral proteinase, in particular, are likely candidates for a regulatory role in intracellular protein degradation *in vivo* since they are active at a near neutral pH and show a high degree of substrate specificity.

It was of interest to examine neutral proteinase activity in animals subjected to the trauma of thermal injury since injury is known to cause a negative nitrogen balance and skeletal muscle is believed to contribute to this loss of nitrogen [6, 22]. A change in muscle protein synthesis and/or degradation after injury might be reflected by altered neutral proteinase activity. In this investigation, total neutral proteinase activity and Ca^{2+}-activated neutral proteinase activity were measured in

[1] The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

[2] In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Revision of the Guide for Laboratory and Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council.

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skeletal muscle from thermally injured animals to determine whether these proteinases played a role in the altered protein metabolism previously observed after thermal injury.

METHODS

Animal care and treatment. Male Sprague–Dawley rats were maintained on a diet of Purina laboratory chow and water provided *ad libitum* and exposed to a 12:12-hr light–dark cycle. The animals were divided into two groups: a sham control group and a burned group. The animals were burned using the procedure described by Herndon *et al.* [8]. Briefly, this procedure consists of anesthetizing the rat (50 mg pentobarbital/kg), shaving the area to be burned, placing the animal in a body mold which exposes a known percentage of the total body surface (TBS), and scalding the exposed area in water to produce the desired wound depth. In this experiment, the rats (350–370 g) received a 60% TBS burn (30% on the dorsum and 30% on the abdomen). To produce a full-thickness wound and minimize damage to underlying tissues, the dorsum was scalded for 9 sec and the abdomen for 3 sec in 98°C water. Saline (30 ml) was given intraperitoneally prior to scalding the abdomen to provide protection to the viscera and to aid in the resuscitation of the animal. The sham control group was anesthetized and shaved. Animals from each group were sacrificed at 3 and 21 days postinjury. All experiments were performed at the same time of day.

Tissue sampling and processing. The soleus and gastrocnemius muscles were removed from anesthetized rats on the specified postburn day. The soleus is classified as an intermediate-fiber type muscle (intermediate oxidative capacity, low glycolytic capacity), and the gastrocnemius is a mixed-fiber type muscle (50% of the fibers are "white," the remainder intermediate and red fibers). These muscles were chosen to examine the proteolytic rate in muscles of differing fiber composition. The muscles were dissected free of connective tissue, minced on ice, and weighed prior to dilution for homogenization. A 10% (w/v) homogenate was prepared from each muscle with 150 m*M* KCl (pH 7.0) using an Ultra-Turrax homogenizer (Tekman Ind., Cincinnati, Ohio). The whole homogenate was stored at −80°C until analysis, at which time all samples were analyzed simultaneously.

Proteinase activity. Neutral proteinase activity was determined for each sample using the assay procedure described by Kar and Pearson [14]. Neutral proteinase activity in the absence of Ca^{2+} was determined by subtracting a blank containing no homogenate sample from a tube containing substrate and homogenate sample without Ca^{2+}. The Ca^{2+}-activated neutral proteinase was determined by measuring the difference in absorbance between assay tubes after incubation with and without Ca^{2+}. Final concentrations of assay reagents were 50 m*M* Tris buffer (pH 7.0), 2 m*M* EDTA (in assay tubes without Ca^{2+}), 2 mg/ml casein-yellow (Calbiochem, La Jolla, Calif.), 1 m*M* $CaCl_2$ (in assay tubes for Ca^{2+}-stimulated proteinase activity), and 20 mg wet wt of sample from a 10% whole homogenate. Whole-homogenate samples were used since preliminary studies showed that approximately 60% of the Ca^{2+}-stimulated neutral proteinase activity was found in the $800g_{(max)}$ pellet after centrifugation. The incubation of the assay tubes was performed in a shaking water bath at 37°C for 20 hr. The activity of Ca^{2+}-activated proteinase was approximately 46% of the final activity after 10 hr of incubation, indicating that the assay approximates linearity over the 20 hr of incubation. After incubation the reaction was stopped by the addition of perchloric acid yielding a final concentration of 5%. The precipitates were settled by centrifugation at 1000*g* for 10 min and the supernatants were read directly at 295 nm on a Beckman DU-8 spectrophotometer.

Statistical analysis was performed using analysis of variance.

RESULTS

Body weight, muscle weight, and muscle-weight to body-weight ratios. On the third

postburn day, no significant changes in body weight or muscle weight were observed (Table 1). By 21 days after injury, the burned group of animals had lower (15%) body weights than sham controls and lower soleus weights (12%). However, the soleus-weight to body-weight ratios were statistically equal between groups, indicating that the decrease in muscle weight was proportional to the decrease in body weight after injury (Table 1).

Proteolytic activity. The soleus had higher rates of neutral proteinase activity (1.5 fold) and Ca^{2+}-activated neutral proteinase activity (1.7 fold) in comparison to the gastrocnemius muscle (Table 2). The neutral proteinase activity in the absence of Ca^{2+} decreased in the soleus (50%) and gastrocnemius (46%) on the third postburn day (Table 2). By 21 days after injury the neutral proteinase activity in both muscles returned to control values. The Ca^{2+}-activated neutral proteinase activity was not altered in either muscle at 3 or 21 days postburn (Table 2). These results indicate that on the third postburn day, neutral proteinase activity is depressed significantly, but Ca^{2+}-activated neutral proteinase is unaltered in both muscles (Table 2). By 21 days after injury, neutral proteinase activity (without Ca^{2+}) and Ca^{2+}-activated neutral proteinase activity are within control levels (Table 2).

DISCUSSION

Although the role played by intracellular proteinases in protein synthesis and degradation is not clear, alterations in neutral proteinase activity have been observed during skeletal muscle disease, such as muscular dystrophy [14], and during immobilization [12] and denervation [13]. Due to their substrate specificity, pH optimum, intracellular localization, and low activity, neutral proteinases are a possible point of regulation for protein degradation.

In this investigation, neutral proteinase activity was measured in the absence and presence of Ca^{2+}. The proteolytic substrate used was casein-yellow, a nitrated casein soluble at physiological pH. These results indicate that neutral proteinases degrade casein-yellow in the absence of Ca^{2+} and that degradation is enhanced in the presence of Ca^{2+}. Casein-yellow is one of the few artificial proteolytic substrates that is degraded by a Ca^{2+}-activated neutral proteinase; hemoglobin and myoglobin do not demonstrate enhanced proteolytic degradation in the presence of Ca^{2+} [14]. Since Ca^{2+}-activated neutral proteinase activity was of interest in this study because of its ability to degrade troponin protein from myofibrils preferentially [2, 4], casein-yellow was employed as the substrate since it can be degraded by Ca^{2+}-activated neutral proteinase.

The observations presented in this study represent results from an *in vitro* assay system. Obviously, the physiological substrate for muscle proteinases is not casein-yellow and the intracellular Ca^{2+} levels do not reach 1 m*M* *in vivo*. Therefore, these results should

TABLE 1

BODY WEIGHTS, SOLEUS MUSCLE WEIGHTS, AND SOLEUS MUSCLE-WEIGHT TO BODY-WEIGHT RATIOS

	Body weight (g)	Soleus weight (mg)	Soleus weight:Body weight ratio (mg/g)
3 Days postburn			
Sham	450 ± 17	150 ± 18	0.35 ± 0.07
Burned	433 ± 5	158 ± 7	0.36 ± 0.04
21 Days postburn			
Sham	499 ± 17	165 ± 5	0.33 ± 0.02
Burned	429 ± 14[a]	148 ± 6[a]	0.34 ± 0.03

Note. Values are $\bar{X}$ ± SEM.
[a] $P < 0.05$ vs sham.

TABLE 2

PROTEOLYTIC ACTIVITY IN RAT SKELETAL MUSCLE AFTER THERMAL INJURY

Group	Neutral proteinase activity (No Ca^{2+})	Neutral proteinase activity (1 m*M* Ca^{2+})	Ca^{2+}-activated neutral proteinase
Sham controls			
Soleus (7)	0.421 ± 0.018	0.957 ± 0.065	0.536 ± 0.050
Gastrocnemius (7)	0.282 ± 0.008	0.593 ± 0.032	0.311 ± 0.026
3 Days postburn			
Soleus (7)	0.280 ± 0.021[a]	0.847 ± 0.073	0.567 ± 0.054
Gastrocnemius (7)	0.193 ± 0.019[a]	0.472 ± 0.043	0.279 ± 0.026
21 Days postburn			
Soleus (7)	0.390 ± 0.020	1.048 ± 0.082	0.658 ± 0.064
Gastrocnemius (7)	0.225 ± 0.016	0.507 ± 0.033	0.282 ± 0.019

Note. Values are $\bar{X}$ ± SEM, *N* per group in parentheses. Activities represent the change in absorbance of acid extract at 295 nm produced by incubating 20 mg wet wt of sample at 37°C for 20 hr (abs. units/20 hr/20 mg). Proteolytic substrate was casein-yellow.

[a] $P < 0.05$ vs sham controls.

not be interpreted literally with regard to the rate of proteolysis *in vivo*. The measured proteinase activities, however, do represent the relative proteolytic capacities between experimental groups.

The results of this investigation indicate that the injured rats had no increase in body weight or muscle weight during the postburn period, while sham-treated control animals gained body weight and muscle weight. The muscle-weight to body-weight ratios remained constant during the postburn period in both experimental groups, indicating that the changes in muscle weight were proportional to changes in body weight. Since muscle weight fluctuates with injury and disease, it is probable that muscle protein synthesis and/or degradation is altered. Other investigators have shown that excess nitrogen is lost from muscle after injury [6]; these alterations are believed to be due to changes in the rates of protein synthesis and/or degradation [3, 7].

These results further show that the soleus has higher levels of neutral proteinase and Ca^{2+}-activated neutral proteinase activities than the gastrocnemius. This could indicate that muscles which derive most of their energy by aerobic means, or are metabolically more active over an extended period of time, require higher levels of proteinases to aid in the degradation of protein from pools which are turning over at faster rates.

Neutral proteinase activity in the absence of Ca^{2+} decreased significantly on the third postburn day in both muscles and returned towards normal by 21 days postinjury. Such neutral proteinase activity is believed to be due to an enzyme(s) other than Ca^{2+}-activated neutral proteinase [5], and this enzyme is thought to degrade sarcoplasmic proteins other than myofibrillar protein preferentially [5].

Burn injury causes physiological alterations which preferentially affect these neutral proteinases. The decrease in neutral proteinase activity implies that (1) neutral proteinase enzyme is lost from the sarcoplasm after burn injury, (2) neutral proteinase activity is preferentially inhibited in the burned group, or (3) decreased enzyme synthesis or increased degradation occurs after injury. Perhaps the decrease in proteolytic activity observed at 3 days postinjury is an effort to spare muscle protein during the first few days after injury, prior to an increase in selected enzyme proteins which is observed during the hypermetabolic period after thermal injury.

By 3 days postinjury the rat has recovered from the initial shock associated with thermal injury and is believed to be experiencing alterations in metabolism which eventually

cause a state of hypermetabolism by 7–13 days postburn [8]. Previous work has shown that selected mitochondrial and sarcoplasmic enzyme levels are elevated in rat muscle during the hypermetabolic period [16]. The decrease in neutral proteinase activity on the third day after injury might be one of the initial intracellular alterations prior to the increase in selected muscle enzymes observed during the hypermetabolic period.

Although blood flow measurements were not performed in this study, previous work from this laboratory has shown that peripheral blood flow to uninjured tibialis anterior muscles of burn patients is unaltered [23]. Other investigators have correlated increases in oxidative enzymes with increased capillary:fiber ratios indicating blood flow may be enhanced under conditions of increased muscle oxidative metabolism. Whether blood flow is altered in association with changes in muscle proteinases is unknown.

This investigation has demonstrated that muscle from thermally injured rats has decreased neutral proteinase activity at 3 days postburn, but that activity returns toward normal by 21 days postinjury. The mechanism responsible for this decrease in activity at 3 days postinjury is unknown.

REFERENCES

1. Bird, J. W. C., Carter, J. H., Triemer, R. E., Brooks, R. M., and Spanier, A. M. Proteinases in cardiac and skeletal muscle. *Fed. Proc.* **39**: 20, 1980.
2. Busch, W. A., Stromer, M. H., Goll, D. E., and Suzuki, A. Ca^{2+}-specific removal of Z lines from rabbit skeletal muscle. *J. Cell Biol.* **52**: 367, 1972.
3. Crane, C. W., Picou, D., Smith, R., and Waterlow, J. C. Protein turnover in patients before and after elective orthopaedic operations. *Brit. J. Surg.* **64**: 129, 1977.
4. Dayton, W. R., Goll, D. E., Zeece, M. G., Robson, R. M., and Reville, W. J. A Ca^{2+}-activated protease possibly involved in myofibrillar protein turnover: Purification from porcine muscle. *Biochemistry* **15**: 2150, 1976.
5. Drabikowski, W., Gorecka, A., and Jakubiec-Puka, A. Endogenous proteinases in vertebrate skeletal muscle. *Int. J. Biochem.* **8**: 61, 1977.
6. Fleck, A., and Munro, H. N. Protein metabolism after injury. *Metabolism* **12**: 783, 1963.
7. Fulks, R. M., Li, J. B., and Goldberg, A. L. Effects of insulin, glucose, and amino acids on protein turnover in rat diaphragm. *J. Biol. Chem.* **250**: 290, 1975.
8. Herndon, D. N., Wilmore, D. W., and Mason, A. D., Jr. Development and analysis of an animal model simulating the human postburn hypermetabolic response. *J. Surg. Res.* **25**: 394, 1978.
9. Holmes, D., Parsons, M. E., Park, D. C., and Pennington, R. J. An alkaline proteinase in muscle homogenates. *Biochem. J.* **125**: 98P, 1971.
10. Houston, R. B., and Krebs, E. G. Activation of skeletal muscle phosphorylase kinase by Ca^{2+}: Identification of the kinase activating factor as a proteolytic enzyme. *Biochemistry* **7**: 2116, 1968.
11. Iodice, A. A., Chin, J., Perker, S., and Weinstock, I. M. Cathepsins A, B, C, D and autolysis during development of breast muscle of normal and dystrophic chickens. *Arch. Biochem. Biophys.* **152**: 166, 1972.
12. Jakubiec-Puka, A., and Drabikowski, W. Changes in proteolytic activity in muscle of rat after immobilization. In I. Hausmanowa-Petrusewicz and H. Jedrzejowska (Eds.), *Structure and Function of Normal and Diseased Muscle and Peripheral Nerve.* Warsaw: Polish Medical Publ., 1974. Pp. 93–97.
13. Jakubiec-Puka, A., and Drabikowski, W. Influence of denervation and reinnervation of autolytic activity and on protein composition of skeletal muscle in rat. *Enzyme* **23**: 10, 1978.
14. Kar, N. C., and Pearson, C. M. A calcium-activated neutral protease in normal dystrophic human muscle. *Clin. Chim. Acta* **73**: 293, 1976.
15. Kohn, R. R. A proteolytic system involving myofibrils and a soluble factor from normal and atrophying muscle. *Lab. Invest.* **20**: 202, 1969.
16. Newman, J. J., Strome, D. R., Goodwin, C. W., Mason, A. D., Jr., and Pruitt, B. A., Jr. Altered muscle metabolism in rats after thermal injury. *Metabolism* **31**: 1229, 1982.
17. Noguchi, T., and Kandatsu, M. Some properties of alkaline protease in rat muscle compared with that in peritoneal cavity cells. *Agric. Biol. Chem.* **40**: 927, 1976.
18. Park, D. C., Parsons, M. E., and Pennington, R. J. Evidence for mast-cell origin of proteinase in skeletal muscle homogenates. *Biochem. Soc. Trans.* **1**: 730, 1973.
19. Park, D. C., and Pennington, R. J. Proteinase activity in muscle particles, *Enzymol. Biol. Clin.* **8**: 149, 1967.
20. Schimke, R. T. Regulation of protein degradation in mammalian tissues. In H. N. Munro (Ed.), *Mammalian Protein Metabolism.* New York: Academic Press, 1970. Pp. 177–228.
21. Schimke, R. T., and Doyle, D. Control of enzyme levels in animals tissues. *Annu. Rev. Biochem.* **39**: 929, 1970.
22. Wilmore, D. W. Hormonal responses and their effect on metabolism. *Surg. Clin. North Amer.* **56**: 999, 1976.
23. Aulick, L. H., Wilmore, D. W., Mason, A. D., Jr., and Pruitt, B. A., Jr. Muscle blood flow following thermal injury. *Ann. Surg.* **188**: 778, 1978.